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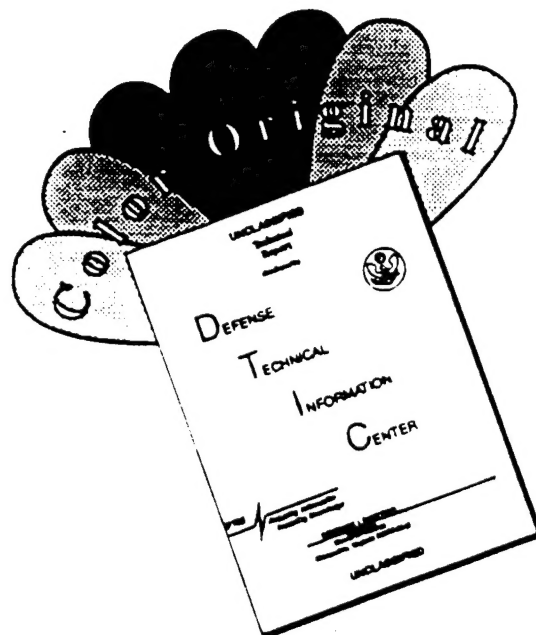
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FOREWORD

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Introduction:

Our goal is to understand the function of the tumor-associated mucin, MUC1, in the progression of cancer in the mammary gland. MUC1 is highly expressed by the majority of cancers and, in particular, by >92% of primary and metastatic breast cancers. The MUC1 protein is a large, rod-like molecule that projects far from the cell surface as a long filament. The protein core is extensively glycosylated through O-glycosidic linkage to serine and threonine, with as much as 50 to 90% of its molecular mass made up of oligosaccharide side chains. This contributes significantly to the rigidity of the molecule. MUC1 is expressed on normal epithelial tissues at low levels. Appearance of MUC1 correlates closely with epithelial differentiation in various organs and is detected well before the organs are functional. The presence of the large, highly extended molecule of MUC1 on the surface of epithelia suggests a physical barrier protecting the cells. MUC1 may be involved in epithelial morphogenesis, perhaps acting to mask adhesive molecules present on the cell surface and aiding in the formation of a lumen. When epithelial tissues become cancerous, MUC1 expression is increased at least ten fold, and the glycosylation and spatial distribution of the protein at the cell surface are altered. MUC1 in normal polarized epithelia is expressed only at the apical side of lumens and ducts. However, in many adenocarcinomas polarization is lost, and the protein is found over the entire surface of the cells. Our hypothesis is that expression of this protein benefits tumor cells and their metastatic counterparts, perhaps by altering the adhesive properties of cells or by providing a protective layer around cells that may shield them from immune surveillance.

The ability to create mice that possess deficiencies in specific genes is providing important insights into the physiological role played by specific proteins during embryonic and postnatal development and during adult life. The expression pattern of Muc-1 in the adult and embryo of the mouse is similar to that of the human (the human gene designation is MUC1; the mouse gene is Muc-1). Muc-1 expression is also elevated in mouse mammary gland tumors. Since mammary gland cancer in the mouse closely resembles human breast cancer and expression patterns are similar, our experiments should enable us to analyze the functional role of Muc-1 in development and in the progression of cancer. To investigate the biological function of the Muc-1 protein we disrupted the Muc-1 gene using homologous recombination in mouse embryonic stem cells. Mice were generated that lacked any expression of the Muc-1 protein. We and others had postulated that Muc-1 on the apical surface of differentiating epithelial cells may repel adjacent cells or mask adhesive molecules, thus promoting the formation of a lumen. However, we were surprised to find that, despite the widespread expression of Muc-1 during epithelial organogenesis, mice lacking Muc-1 were born at the expected frequency and appeared normal in all respects.

Direct evidence of a role for MUC1 in the development and progression of breast cancer has not been demonstrated previously. In many cancer cells polarization of the epithelial cells is lost and the MUC1 protein can be detected on all cell surfaces, including those facing the stroma and adjacent cells. Under these circumstances, the anti-adhesive property of MUC1 may destabilize cell-cell and cell-substratum interactions, thus promoting the disaggregation of a tumor site, leading to tumor spread and metastasis. Previous studies have suggested various possible roles for the MUC1 mucin in facilitating tumor growth, including inhibition of cell-cell contacts, protection from recognition and destruction by immune cells, and also serving as an E-selectin ligand to facilitate escape of metastatic cells from the blood stream. Thus overexpression of the Muc-1 molecule could provide many potential benefits to tumor cells.

We are currently using the Muc-1 deficient and control mice to investigate the role of the Muc-1 molecule in normal development and in the development and progression of breast cancer in mice. Three specific aims have been proposed:

Specific Aim 1: Analysis of the effects of Muc-1 gene mutations on epithelial organogenesis. Chimeric mice mutant for the Muc 1 gene will be crossed with 129/SV mice and the heterozygous progeny intercrossed to produce homozygous mutant mice on a pure 129/SV background. F1 heterozygotes will be backcrossed onto C57Bl/6 line through 12 generations to produce homozygous mutant mice on a pure C57Bl/6 background. Histological analysis will be conducted on embryos and adult mice, using both mutant and control mice of the inbred strains 129/SV and C57Bl/6. Both strains will be studied to identify variability that may be due to strain differences.

Specific Aim 2: Analysis of the effects of Muc-1 gene mutation on tumor formation and progression. Mice carrying the mutant Muc-1 alleles will enable us to test the effects of mucin deficiency on tumor development. Tumors will be elicited in mutant and control mice using several different methods, to rule out anomalies that may occur if only a single type of tumor is studied. The systems chosen include mating Muc-1 mutant mice with transgenic mice that develop mammary gland tumors and eliciting tumors with chemicals. The transgenic mice carry either the polyoma virus middle T antigen or the unactivated c-neu oncogene under the transcriptional control of the mouse mammary tumor virus (MMTV) long terminal repeat. One hundred percent of polyoma virus middle T antigen transgenic females develop mammary tumors by 4 months of age (Guy et al., 1992a). Fifty percent of the neu transgenic females develop tumors by the age of 205

days, with some tumors occurring as early as 4 months of age (Guy et al., 1992b). Carcinogen induced mammary tumors will be produced by the injection of N-methyl-N-nitrosourea (MNU) and medroxyprogesterone acetate (MPA). Mice treated with both MNU + MPA showed 79% incidence with a latency of 154 ± 19 days (Pazos, 1991).

Specific Aim 3: Analysis of the effect of Muc-1 gene deletion on metastasis of mammary gland tumors. The transgenic mouse expressing the c-neu oncogene develops mammary gland tumors of which about 72% metastasize to the lungs (Guy et al., 1992b). This mid-range percentage of metastasis development should enable us to determine if a lack of the Muc-1 mucin alters the percentages. These studies will be performed on the same animals being used in the tumor formation and progression studies (specific aim 2); when the animals are euthanized in order to analyze the tumor, the tissues in the mouse will be carefully examined for metastatic lesions. The number of lesions, the percentage of tumors that metastasize, and the organ specificity of the metastatic lesions will be determined.

Body:

Specific Aim 1: Analysis of the effects of Muc-1 gene mutations on epithelial organogenesis.

Mice homozygous for the Muc-1 mutation have been produced and have been demonstrated to be healthy, fertile and viable. Over 400 offspring of heterozygous intercrosses were screened in outbred mice (C57Bl/6 x 129SV). Homozygous animals were identified through a PCR-based screening procedure and results were initially confirmed through Southern blotting of EcoRI-digested tail DNA utilizing the 5' flanking probe. In all cases, animals homozygous for the disrupted Muc-1 allele (-/-) were obtained at the expected Mendelian frequency of 1:2:1 (Wild type : Heterozygotes : Homozygous Mutants). In addition, inbred 129SV heterozygotes were obtained from the original chimeric animals and intercrossed to derive an inbred line homozygous for the Muc-1 mutation. Similarly, inbred C57Bl/6J heterozygotes are being derived through a series of backcrosses onto C57Bl/6J. These mice are currently at N8 (99% inbred with respect to C57Bl/6J). Homozygous Muc-1 mutant mice of the 129SV and C57Bl/6J line (N8) have been produced and appear healthy and viable.

In order to determine whether the insertion of the LacZ-pgk neo cassette resulted in the efficient disruption of Muc-1 transcription and subsequent translation, total RNA was prepared from a panel of tissues isolated from +/+, +/- and -/- litter mates. Approximately equivalent amounts of total RNA were subjected to northern analysis with the previously characterized Muc-1 cDNA probe, pMuc2TR (Spicer et al., 1991). Expression of Muc-1 was found to be reduced in the tissues of heterozygous mice and undetectable in homozygous mice (Fig 1). In addition, immunohistochemistry indicated no detectable Muc-1 protein on the apical surface of Muc-1 -/- secretory epithelial tissues (Fig 2). Thus, targeted inactivation of the Muc-1 gene by the replacement vector, 129Muc-1GT, resulted in the creation of a null Muc-1 allele.

Mice deficient in Muc-1 were obtained at the expected frequency from all crosses. These mice appeared to develop normally and gained weight at the same rate as their heterozygous and wild-type litter mates (data not shown). Examination of hematoxylin-eosin stained sections prepared from all the major organs revealed no obvious differences between Muc-1 deficient mice and their corresponding litter mates (data not shown). Similarly, whole mounts of virgin mammary glands of twelve week old wild-type and Muc-1 null animals showed no obvious differences in glandular morphology (data not shown). All possible pairwise crosses of genotypes indicated no differences in the fertility of the parents, subsequent litter size, growth rate and survival of the litters (data not shown). This would suggest that Muc-1 present in milk is not important for the growth and survival of neonates under pathogen-free conditions.

We explored the possibility that the up-regulation of expression of one or more mucin-like genes or membrane glycoproteins may have accounted for the apparent lack of a phenotype in Muc-1 deficient mice. Probes were obtained either as antisense oligonucleotides (50mers) or, alternatively, cloned cDNAs were utilized (Table 1). Total RNAs isolated from +/+, +/- and -/- litter mates were investigated by slot blot analyses with the various probes. No difference in expression levels was observed for Muc-2, or Muc-4, although high levels of Muc-4 expression were observed in lactating mammary gland, salivary gland, lung, stomach, kidney, and colon. Similarly, no difference was observable for other mucin-like genes, including ASGP-2, CD34, CD43 (leukosialin), glycophorin and MadCAM-1. The expression level of GlyCAM-1 was elevated in several outbred homozygous animals, but this apparent increase in expression was not consistent in inbred homozygotes, nor did it appear to correlate with an increase in GlyCAM-1 protein levels in milk. In addition, no difference was observable in the expression of thrombospondin-3 (Thbs-3), although the expression of this gene did appear to be highly variable in the tissues tested. This is important as Thbs-3 is located only 2.5 Kbases upstream from the Muc-1 gene and its' expression could potentially have been altered by the mutation of Muc-1.

Although lack of the Muc-1 molecule does not appear to significantly affect the development, viability or fertility of homozygous Muc-1 mutant mice, it is possible that subtle changes in organogenesis are occurring. To address this possibility, future studies will assess the effect of mutation of the Muc-1 gene on organogenesis in inbred mice of the 129SV/j and C57Bl/6 strains using hematoxylin and eosin stained sections for morphology and special stains (i.e., PAS stain for mucins, alcian blue cationic dye for glycosaminoglycans which also delineates the epithelial stromal boundary, Masson's trichrome and Sirius red F3BA stains for collagen) will be utilized as needed.

Specific Aim 2: Analysis of the effects of Muc-1 gene mutation on tumor formation and progression.

To investigate the role of Muc-1 in tumor development and/or progression, we compared the growth of mammary tumors in Muc-1 $-/-$ and $+/+$ mice. We chose to start our study of the role of Muc-1 in tumor formation using a transgene that elicited tumors at a more rapid rate than the neu protooncogene. We utilized mice transgenic for the polyoma virus middle T antigen (Guy et al., 1992a). The middle T antigen is under the control of the mouse mammary tumor virus (MMTV) promoter and, in female mice, expression is specific for the mammary gland and to a lesser extent the salivary gland. Virgin female mice of this strain have been shown to develop multifocal breast tumors by 2 months of age and by 4 months of age greater than 50 percent of these mice will have developed lung metastases. Interestingly, the middle T antigen has been demonstrated to require the presence of the src oncogene for its ability to transform mammary cells (Guy et al., 1994). Similarly, it has been demonstrated that the neu oncogene, implicated in up to 30 percent of human breast cancers (Slamon et al., 1987), binds to and activates src tyrosine kinase activity (Muthuswamy et al., 1994). We have employed the middle T oncogene in this study due to its rapid time course of tumor induction, reliable production of spontaneous tumor metastases, and the possible commonality of signal transduction pathways with the neu protooncogene.

For the study, 85 female Muc-1 $-/-$ mice and 35 female Muc-1 $+/+$ mice were utilized. All mice were virgin females positive for the polyoma virus middle T antigen transgene. Mice were housed in groups of 5 mice per cage and palpated 3 times per week, starting at 60 days and continuing through 124 days. Fifty percent of mice in this study developed palpable lesions of the mammary gland by 68 days of age. There was no significant difference in the rate of appearance of palpable lesions between Muc-1 mutant and wild type mice. By 4 months of age, tumors appeared in 100% of wild-type mice and in 98% of mutant mice. Tumors in Muc-1 mutant and

wild type mice had similar histological appearances and were poorly differentiated adenocarcinomas (Fig. 3A). Pathological analysis showed that the tumors were high grade, based on the high mitotic rate, the solid growth pattern, and the presence of central necrosis. Immunohistochemical analysis using antiserum directed to the Muc-1 cytoplasmic tail showed that tumors that developed in Muc-1 +/+ animals expressed high levels of Muc-1 (Fig. 3B). Interestingly, tumor growth rate differed significantly between the two groups (Fig. 3C). As early as 104 days of age, Muc-1 -/- mice had significantly smaller tumors than did mice with wild type Muc-1 alleles ($p < 0.05$) and by the 124 day endpoint, differences in tumor size were highly significant ($p < 0.001$) (two-sample t-test). These studies are the first to directly implicate the Muc-1 molecule in the facilitation of mouse mammary tumor growth.

Specific Aim 3: Analysis of the effect of Muc-1 gene deletion on metastasis of mammary gland tumors.

To investigate whether the Muc-1 molecule plays a significant role in facilitating tumor metastasis, Muc-1 mutant and wild type mice were crossed with transgenic mice expressing the polyoma virus middle T antigen under the control of the mouse mammary tumor virus promoter. As mentioned previously, 85 Muc-1 -/- female mice and 35 Muc-1 +/+ female mice were examined. Mice were housed in groups of 5 and palpated 3 times per week from day 60 to day 124. Mice were terminated on day 124 and their organs examined for the presence of metastases. As was previously reported (Guy et al., 1992a), only the lungs contained metastatic tumor foci. At termination, the lungs were removed, fixed in methacarn and scored for grossly observable lung metastases under the dissecting microscope. Overall, 58% of mice developed grossly observable lung metastases, with 53% of Muc-1 -/- mice and 67% of Muc-1 +/+ mice developing metastases (Fig. 3D). Although this difference suggests a trend towards decreased rates of tumor metastasis in Muc-1 -/- mice, it was not statistically significant as assessed by chi-square analysis ($p > 0.12$). However, based on the sample sizes in this study, the power to statistically detect the observed difference was only 33%. It is possible that with a larger sample size, this difference in metastatic rate would be statistically significant.

Conclusions and Future Studies:

The present studies demonstrate that the Muc-1 molecule has been successfully mutated in mice and that no deleterious effects were observed in mice of two different strains. These data suggest that Muc-1 may not play a crucial role in organogenesis. Alternately, it is possible that

other transmembrane or extracellular molecules are upregulated to compensate for the lack of Muc-1 in the developing embryo. Current studies failed to indicate upregulation of mRNA for other known mucin like genes. However, it remains possible that other molecules may be upregulated to compensate for the lack of the Muc-1 gene. Although not proposed as part of this project, the upregulation of other molecules to compensate for the loss of Muc-1 expression be assessed will in our laboratory using differential display PCR technology in inbred mice.

This study demonstrates for the first time that the Muc-1 molecule facilitates growth of breast tumors in mice transgenic for the polyoma virus middle T antigen. Tumor growth rate was significantly decreased in mice homozygous for the Muc-1 mutation when compared with wild type control mice. To insure that this finding is not specific to tumors induced by the polyoma virus middle T antigen oncogene, two other tumor induction systems will be examined. A study to investigate the effect of Muc-1 gene mutation on tumor development and progression induced by the neu protooncogene is currently being initiated. The neu transgenic mouse is a useful model for several reasons. It is estimated that 30 percent of human breast cancers overexpress the neu protooncogene and these transgenic mice have long tumor latencies similar to that observed in humans. neu transgenic mice have been mated to Muc-1 mutant and wild type mice and breeding is currently underway to produce neu transgenic mice homozygous for the Muc-1 mutation or wild type Muc-1 genes. Presently, 20 neu transgenic female mice of Muc-1 $-/-$ and $+/+$ genotype have been produced. Pilot experiments suggest that tumor latencies should be approximately 8 to 10 months in virgin females. Based on this pilot study and the previous polyoma virus middle T antigen induced tumor study, target numbers for the experiment will be 80 females per group which should provide statistically significant results. A second tumor model utilizing chemical carcinogenesis, induced by the injection of MNU + MPA, will also be investigated. The combination of these 3 experimental systems should indicate if Muc-1 plays an important role in the development or progression of breast cancer.

Although the present study indicates that Muc-1 overexpression facilitates the growth rate of the primary tumor, the mechanism of this facilitation is not clear. To investigate whether the decrease in growth rate in Muc-1 $-/-$ mice is due to decreased cell proliferation or to increased cell loss, rates of cell proliferation, apoptosis and levels of necrosis will be compared in polyoma virus middle T antigen induced tumors of Muc-1 $-/-$ and $+/+$ mice. Tumors will be palpated 3 times weekly and when they reach a weight of 1 gram, mice will be injected i.p. with 5' Bromodeoxyuridine (BrdU). Mice will be terminated 1 hour following BrdU injection and tumors removed and bisected. Half of the tumor will be fixed in methacarn for histological processing to allow determination of levels of apoptosis and necrosis. The remaining tumor halves will be fixed

in ethanol and single cell suspensions obtained for flow cytometric determination of rates of cell proliferation and apoptosis. Rates of cell division will be scored by measuring the percentage of cells which incorporate BrdU, while rates of apoptosis will be determined by labeling apoptotic cells using biotin labelled dUTP in the terminal deoxynucleotide transferase assay. This experiment should allow the determination of whether the Muc-1 molecule facilitates primary tumor growth rate by increasing the rate of cell proliferation, or by decreasing cell death.

Interestingly, although more Muc-1 +/+ mice developed lung metastases than did Muc-1 -/- mice, this trend towards decreased rates of tumor metastasis in Muc-1 deficient mice did not reach statistical significance. It is possible that the rapid kinetics of the middle T tumor phenotype may have rendered some immune responses impotent. The middle T mice exhibit hyperplastic mammary glands as early as three weeks of age and the rapid production of multifocal mammary adenocarcinomas. In many animals tumors developed in every mammary gland. Further analysis of the role of Muc-1 in metastasis may await a tumor model more relevant to human breast cancer. Such a study is in progress, utilizing mice with the neu protooncogene under control of the MMTV promoter, which develop focal mammary tumors and metastasize after long latency (about nine months). Although very time-consuming, this model may be more biologically relevant. Muc-1 -/- and +/+ mice transgenic for the neu protooncogene (from specific aim 2) will be terminated when tumors reach a mass of 2 to 3 grams. Lungs will be removed, fixed in methacarn and scored for the presence of metastatic foci under the dissecting microscope. The longer latency for tumor formation and metastasis should accentuate any differences in metastatic potential. Alternately, measurement of grossly evident spontaneous metastasis in mice at 124 days of age, when most mice had well established primary tumors, may not be a sensitive enough measure of tumor metastasis. To address questions regarding the sensitivity of the assay to detect differences in metastatic abilities, a second assay of metastatic ability will also be utilized. Tumor cell lines have been isolated from polyoma virus middle T antigen induced mammary tumors growing in Muc-1 -/- and +/+ mice. These cell lines will be injected into the tail veins of Muc-1 -/- and +/+ mice and the lung colonizing ability of these cell lines compared. This tumor metastasis system has the advantage of controlling for the seeding ability of tumor cells and focuses on the ability of tumor cells to arrest in the target organ, extravasate from the bloodstream and grow in the target organ. These studies should define whether or not overexpression of the Muc-1 molecule facilitates tumor metastasis.

The studies described in this report utilize the unique strength of the Muc-1 mutant mouse model to investigate the role of the Muc-1 molecule in organogenesis, tumor development and progression and in tumor metastasis. These are the first studies to directly demonstrate a role for

Muc-1 overexpression in facilitating the growth of breast cancer *in vivo*. It is hoped that in the long term the data derived from these studies could be used to improve the treatment of human breast cancer.

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Figure Legends:

Figure 1. Northern Analysis of Muc-1 Expression in Muc-1 Deficient Mice.

Approximately equivalent amounts of total RNA isolated from inbred 129SV/J +/+, +/- and -/- mice were size-fractionated through a 1.2% formaldehyde agarose gel and transferred to nylon membrane. RNA was hybridized with the mouse Muc-1 cDNA probe, pMuc2TR. Below is an image of the membrane after staining with methylene blue prior to hybridization to detect ribosomal RNA. The position of the 18S ribosomal RNA is indicated.

Figure 2. Immunohistochemical Investigation of Muc-1 Expression in Muc-1 Deficient Mice.

Tissues from +/+, +/- and -/- mice were isolated, fixed and sectioned. Sections were incubated with polyclonal antiserum to the cytoplasmic tail (CTI) or antiserum previously blocked with immunizing peptide, followed by FITC-conjugated swine anti-rabbit immunoglobulins. Equivalent sections were viewed and photographed under identical conditions. Panels A, D, G, J, M +/+; B, E, H, K, N, +/-; C, F, I, L, O -/-. Panels D-F represent the equivalent areas shown in A-C, the distinction being that the antiserum had been previously blocked with peptide. No Muc-1-associated fluorescence could be detected in -/- tissues. Bar = 200 μ m.

Figure 3. Primary Tumor Growth Rate is Reduced in Muc-1 -/- Mice.

A. Hematoxylin-eosin stained sections of tumors taken at 124 days, showing poorly differentiated adenocarcinomas.

B. Expression of Muc-1 protein was assessed by immunofluorescent staining with a polyclonal antiserum directed against the Muc-1 cytoplasmic tail. Tumors in Muc-1 +/+ mice expressed high levels of Muc-1 protein. Bar (parts A and B) equals 100 microns.

C. Graph showing growth rate of polyoma middle T-induced mammary tumors in Muc-1 -/- (filled square) and Muc-1 +/+ (open circle) mice. At 104 days, Muc-1 -/- mice had significantly smaller

tumors than did Muc-1 +/+ mice ($p < 0.05$). By the 124 day endpoint, differences in tumor size were highly significant ($p < 0.001$). Asterisks indicate statistical significance.

D. Graph showing the percentage of Muc-1 +/+ and -/- mice with metastatic lesions in the lung at 124 days. The trend towards decreased rates of tumor metastasis in Muc-1 -/- mice suggested that the lack of Muc-1 was showing some effects. Our sample size was not sufficiently large to reach statistical significance.

Table 1. List of Genes Screened for in Muc-1 Deficient Mice

Gene	Species	Probe Name/Type	Location
Muc-1	mouse	pMuc2TR	Tandem repeat
Muc-2	rat	VR-1A	5' cDNA
Muc-4	mouse	pMuc7.18/genomic	Tandem repeat
GlyCAM-1	mouse	antisense-oligo	bp 468-419
MadCAM-1	mouse	antisense-oligo	bp 1248-1199
Glycophorin	mouse	antisense-oligo	bp 900-851
PSGL-1	human	antisense-oligo	bp 1061-1015
ASGP-2	rat	antisense-oligo	bp 2175-2126
CD34	mouse	antisense-oligo	bp 1258-1209
CD43	mouse	antisense-oligo	bp 1183-1134
CD44(Pgp-1)	mouse	antisense-oligo	bp 1000-951
CD45(Ly-5)	mouse	antisense-oligo	bp 4250-4201
ESTO1966(Gene Y)	mouse	antisense-oligo	3' cDNA
Thbs-3	mouse	mThbs-3-Eco	2.1 kb EcoRI (3')

Antisense oligonucleotides were 50mers corresponding to sequence close to the 3' end of the published cDNA. If synthesized according to rat or human cDNA sequence, oligos were designed corresponding to sequence coding for a presumed functionally conserved portion of the gene. Base pair references refer to the numbering on the sense strand of the published sequence.

Lactating
Mam. Gland

Colon

Lung

Stomach

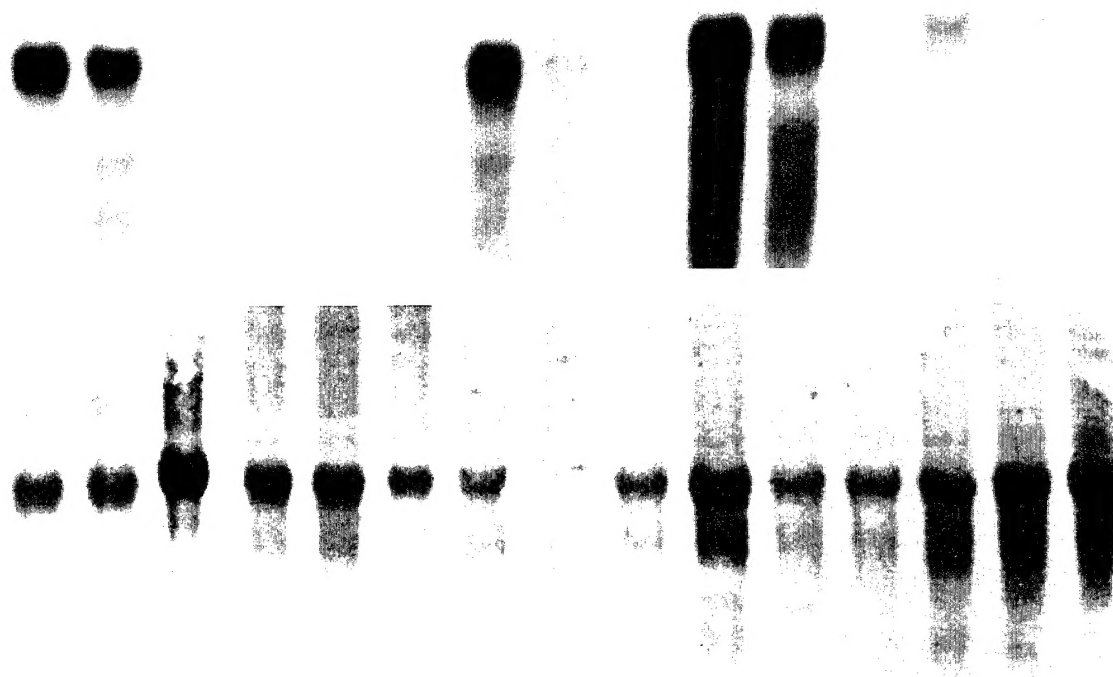
Kidney

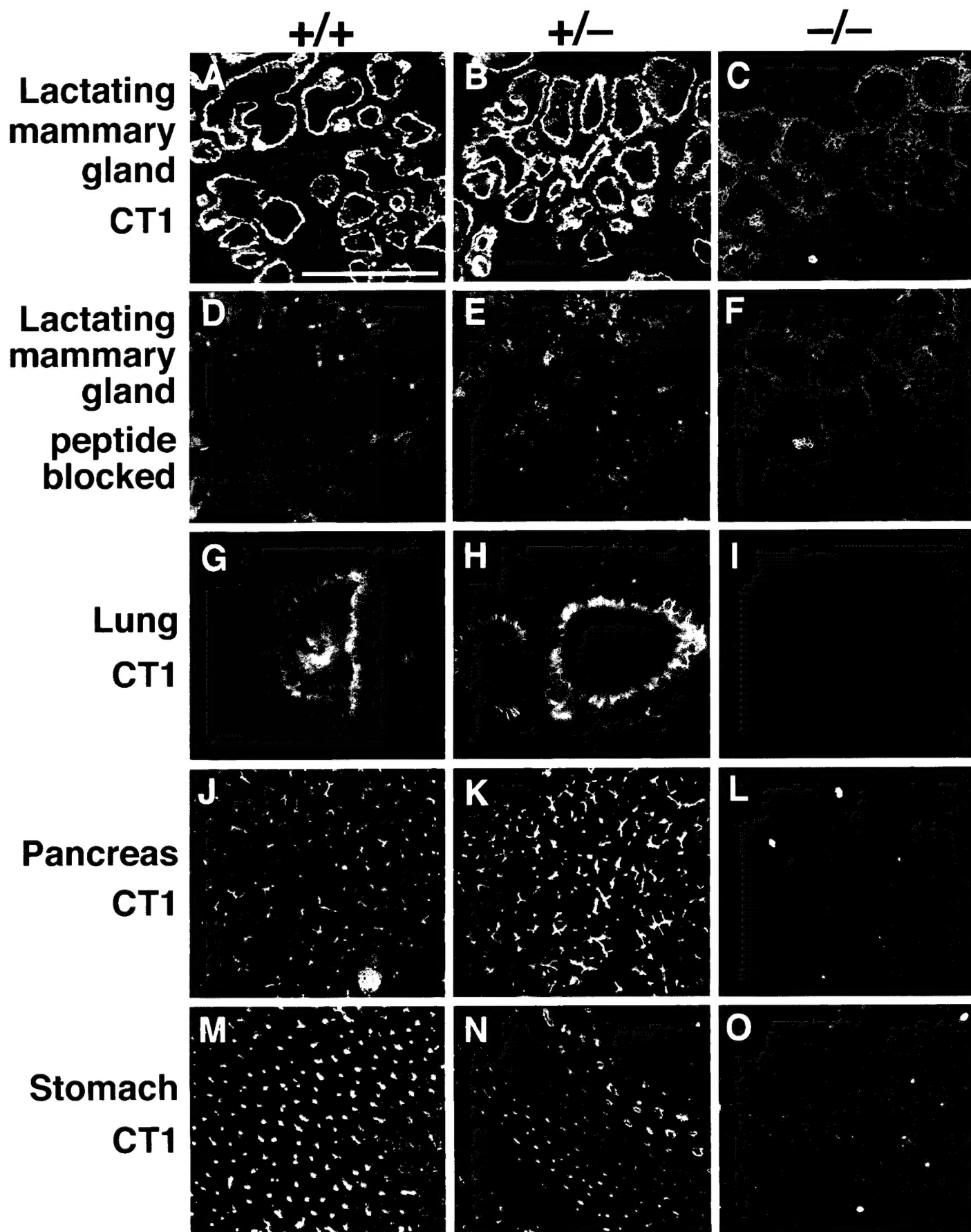
$\frac{+}{+}$	$\frac{-}{+}$	$\frac{-}{-}$	$\frac{+}{+}$	$\frac{-}{+}$	$\frac{-}{-}$	$\frac{+}{+}$	$\frac{-}{+}$	$\frac{-}{-}$	$\frac{+}{+}$	$\frac{-}{+}$	$\frac{-}{-}$	$\frac{+}{+}$	$\frac{-}{+}$	$\frac{-}{-}$
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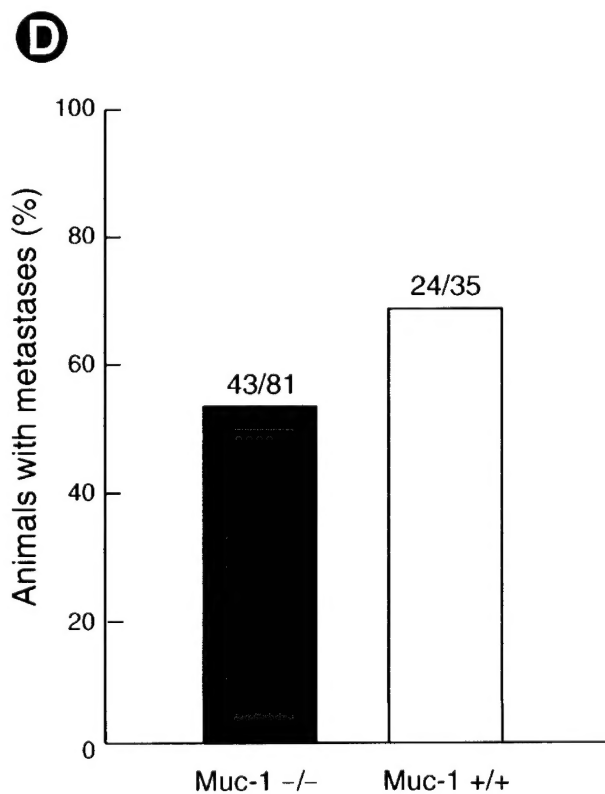
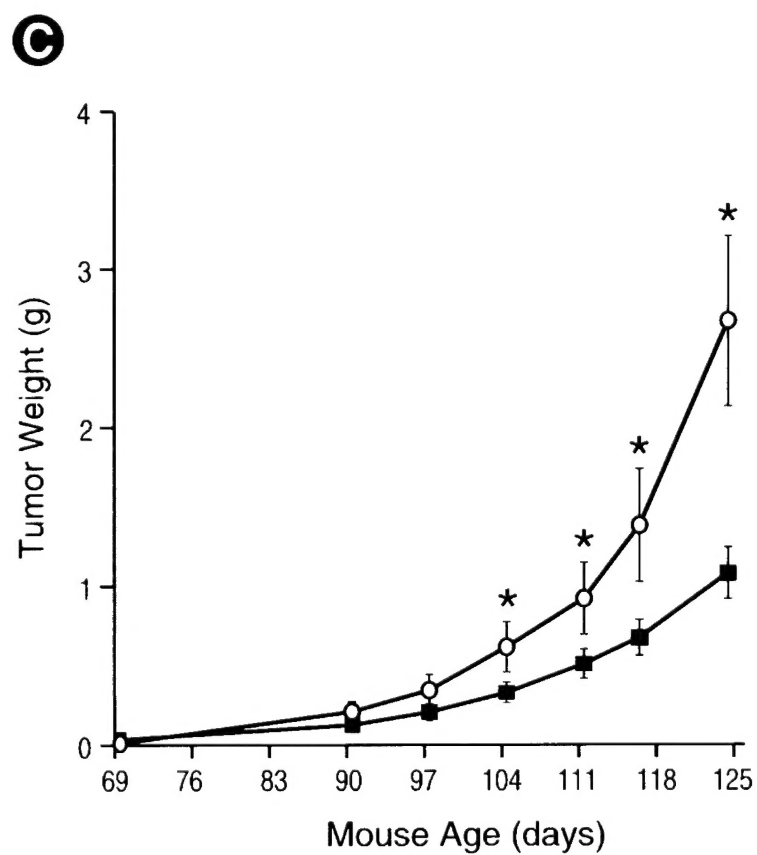
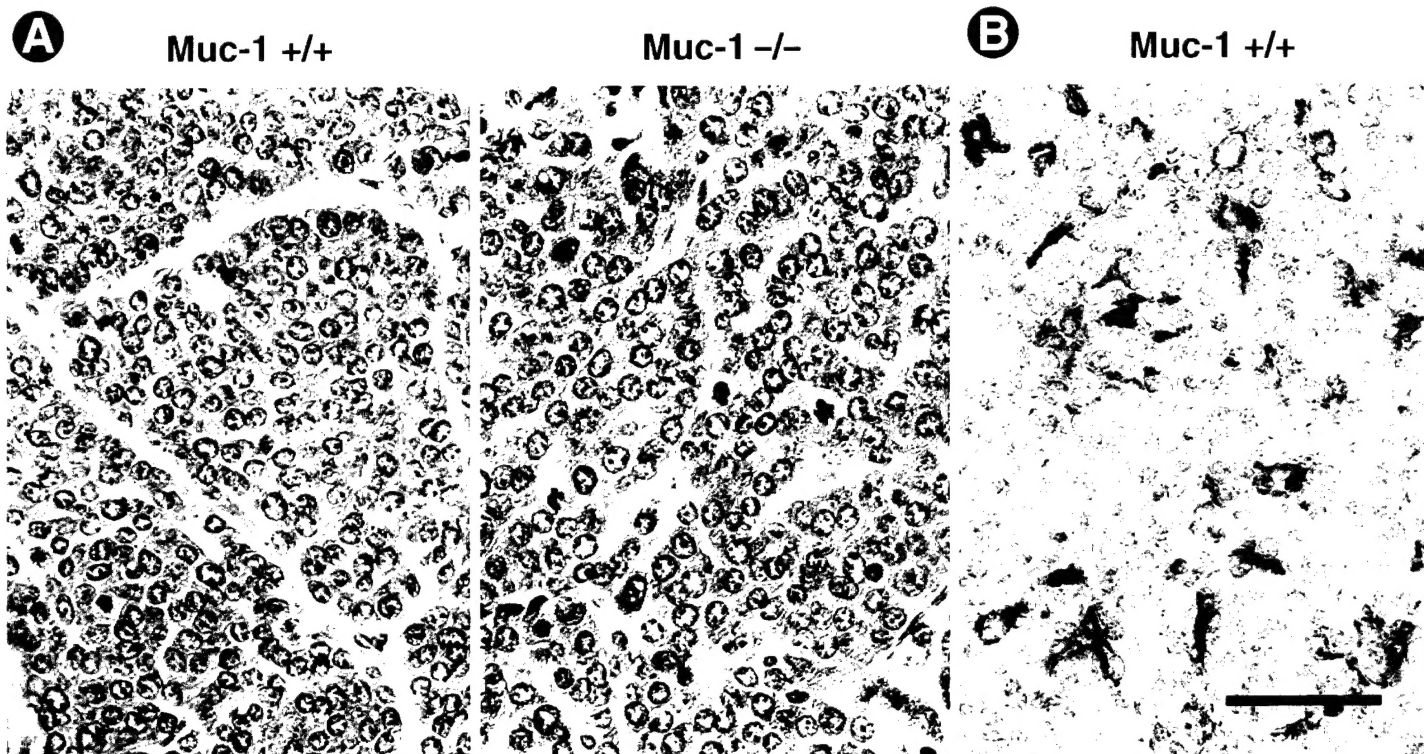
Muc-1

2.2kb

18S
rRNA







Appendix

A paper based on the generation and characterization of the Muc-1 deficient mice and the effect of Muc-1 mutation on the growth of tumors induced by the polyoma virus middle T antigen has been submitted to the Journal of Biological Chemistry. The paper is entitled Delayed Mammary Tumor Progression in Muc-1 Null Mice.